



SCIENTIFIC REPORTS

OPEN

Environmental novelty exacerbates stress hormones and A β pathology in an Alzheimer's model

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Cognitive stimulation has been proposed as a non-pharmacological intervention to be used in primary, secondary and tertiary prevention approaches for Alzheimer's disease. A common familial Alzheimer's disease transgenic model showed heightened levels of the stress hormone, corticosterone. When exposed to periodic enhanced cognitive stimulation, these animals demonstrated further heightened levels of corticosterone as well as increased A β pathology. Hence, Alzheimer's disease may be associated with hypothalamic-pituitary-adrenal (HPA) axis dysfunction, causing stimulatory environments to become stress-inducing, leading to a glucocorticoid-pathology cycle contributing to further A β release and plaque formation. This finding suggests that stimulation-based interventions and local environments for people with Alzheimer's disease need to be designed to minimise a stress response that may exacerbate brain pathology.

The ageing of the global population is accompanied by an increasing substantial social and financial impact of ageing-related diseases that cause dementia. Among elderly populations, Alzheimer's disease (AD) is the most common cause of dementia¹. Research has consistently demonstrated that the environment influences the brain both structurally and functionally over the lifetime². In this respect, among modifiable factors that are associated with a reduced risk of developing dementia, is living a cognitively engaged life. Indeed, those who engage in high levels of cognitive activity have approximately half the risk of developing dementia than those who engage in low levels³. The cognitive reserve theory (CR) posits that a high-level of intellectual engagement over the life promotes strong and efficient neuronal connections that enables the neural system capacity to withstand a greater degree of pathological insult before clinical expression of the disease process emerges⁴.

In animal models, the building of cognitive reserve can be modelled experimentally through an environmental enrichment (EE) paradigm. EE describes the manipulation of an animals' environment in order to generate novelty and complexity, and heighten cognitive, sensory, and physical stimulation⁵. EE applied in early-life has been found to boost cognitive performance in familial AD (FAD) transgenic mouse models to the level of healthy wildtypes (Wt). However, EE is reported to have a variable effect on A β pathological burden in transgenic mice expressing human FAD-related gene mutations, with reported decrease^{6–8}, no change^{9–11}, or increase^{12,13} in A β load. Alternatively, it is possible that more complex, and novel stimulation may be required to buffer AD-related neuropathology³. Further, individuals are more likely to take up interventions to delay dementia onset in mid-to late-life, or even after the onset of early symptoms of dementia. In a previous investigation, we demonstrated some beneficial effects of mid-life EE on cognitive function, with no changes to A β neuropathological burden in FAD model mice¹⁴. Therefore, we sought to examine whether added periodic augmentation of more novel stimulation (EE+) in FAD animals from mid-life (6 months of age), would produce findings consistent with previous reports of reduced A β burden following EE^{6–8}. In order to examine EE in mid to later-life, mice were raised in standard housing (minimal stimulation; SH) conditions until 6 months of age, and then randomly assigned to SH, environmental enrichment (EE) or enhanced environmental enrichment (EE+) conditions, where the animals remained until 12 months of age. Mice in EE lived in a larger cage to that of SH, and enrichment objects were present in the cage (wooden and plastic blocks of differing shapes and sizes, platforms, a ball, running wheel, and a mouse hut). Mice in the EE+ condition lived in EE, but were also exposed to a novel, larger cage three times per

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week that contained novel enrichment objects (e.g. steps, ladders, large running wheel, mazes, toys of different textures; changed weekly).

Results

Mid-life EE+ was associated with increased A β plaque pathology in the hippocampus.

Following the 6-month intervention, when mice were 12 months of age, we hypothesised exposure to the EE+ intervention would potentially be associated with a reduction in A β plaque burden. We analysed fibrillar plaque load by Thioflavin-s (Fig. 1E) staining and by immunostaining of human unaggregated, oligomeric, and fibrillar forms of A β_{1-42} with the MOAB-2 antibody (Fig. 1F), in the neocortex and hippocampus of APP/PS1 mice. No significant alterations to A β load in the neocortex as a function of housing condition was detected for either Thioflavin-s or MOAB-2 labelled A β plaques (Fig. 1A,C). In contrast, there was a large ($d = 1.08$) and significant increase in hippocampal fibrillar A β plaque load in APP/PS1 mice exposed to the EE+ condition (Fig. 1B), and this effect was also detected for A β_{1-42} immunolabelling (Fig. 1D; $d = 1.83$).

Mid-life EE+ was associated with increased A β_{1-42} in neocortex. Next, we examined levels of soluble A β_{1-42} in the neocortex and hippocampus, and found an overall significant effect of housing condition on A β_{1-42} . APP/PS1 mice housed in EE+ showed a large ($d = 1.89$) and significant increase in A β_{1-42} in neocortex, compared to mice from SH or EE (Fig. 2A). However, in contrast to the finding of an increase in A β plaque deposition in the hippocampus, this effect was not observed for A β_{1-42} levels in the hippocampus (Fig. 2B). These data suggest that EE+ is associated with increases in A β levels in the neocortex, and increased deposition of A β in the hippocampus.

APP/PS1 mice demonstrated elevated levels of corticosterone. While our assumption had been that placing animals in an enhanced EE condition may have augmented cognitive and physical stimulation, it was also possible that this exposure had broader physiological effects that may have led to increased A β pathology, and that the FAD background also contributed to an unexpected reaction to the EE+ condition. Previous clinical studies have reported that people living with AD generally have higher blood-levels of the stress hormone, cortisol, when compared to healthy people^{15,16}. However, whether the disease process itself alters normal stress physiology, or whether stress increases vulnerability to developing AD, is unknown. It is widely accepted that stress induces activation of the hypothalamic-pituitary-adrenocortical (HPA) axis, which leads to a subsequent release of cortisol in humans, or corticosterone in rodents, from the adrenal cortex^{17,18}. Several studies, using experimental paradigms designed to induce stress, have demonstrated an accelerated onset, or faster rate of progression of AD, in FAD mouse models^{19–23}. Such evidence led us to hypothesise that the novelty and complexity conferred by the EE+ condition, elevated stress levels, which may in turn have increased A β pathological burden in APP/PS1 mice in EE+ housing. We first compared levels of the stress hormone, corticosterone, between healthy control Wt and APP/PS1 mice from the SH condition, in order to determine whether the presence of the APP/PS1 transgene was associated with elevated corticosterone. Corticosterone levels in serum were significantly increased in 12-month old APP/PS1 mice compared to healthy Wt mice, which represented a large effect ($d = 1.09$) (Fig. 3). This finding supports the hypothesis of HPA axis dysfunction in AD.

Mid-life EE+ was associated with elevated corticosterone levels in APP/PS1 mice. Following the finding of elevated levels of corticosterone in APP/PS1 mice compared to Wt mice from SH, we included EE and EE+ conditions into the analysis. First, we found an overall significant interaction effect of genotype \times housing on corticosterone levels. Due to the APP/PS1 mice demonstrating increased corticosterone levels, we analysed genotypes separately to investigate housing effects. In Wt mice, there was no significant housing condition effect on corticosterone levels. However, there was a significant and large effect of housing condition for APP/PS1 mice. While the APP/PS1 mice from SH exhibited increased corticosterone levels relative to Wts, APP/PS1 mice from EE+ showed further elevated corticosterone levels, being significantly higher compared to APP/PS1 mice from both the SH and EE conditions ($d = 1.05$) (Fig. 3).

Discussion

Our study has shown that periodic augmentation of EE conditions in APP/PS1 mice may have a deleterious effect on A β pathology, and this was associated with heightened levels of corticosterone, in a FAD model that already showed elevated stress hormone levels over Wt animals of a similar age. In contrast, sustained exposure to regular EE conditions did not affect corticosterone levels or amyloid pathology, indicating a potential capacity to habituate to long-term changes in the housing environment. While APP/PS1 animals housed in EE+ had increased A β pathology along with elevated corticosterone, no significant behavioural differences were detected on the Y maze test of spatial short-term memory, or the Barnes maze test of spatial learning and long-term memory relative to APP/PS1 mice housed in SH or EE (data not shown). The finding of increased pathological burden without cognitive dysfunction may offer support for the cognitive reserve theory⁴, however following up at later time points when neural resources are reduced, would be of interest.

Clinical studies have included reports of people living with AD to generally have higher levels of blood cortisol when compared to healthy people^{15,16}. Moreover, elevated levels of corticosterone has been reported previously in a FAD mouse model²⁴, and A β accumulation has been associated with increased sensitivity of the HPA axis²³. The present study confirms HPA axis dysfunction in an animal model of early AD. Several studies have demonstrated using experimental paradigms designed to induce acute stress (e.g. by restraint, loud noise, and bright light) an accelerated onset, or faster rate of progression of AD, in FAD mouse models^{19–23}. However, in the current report, we found an association between the periodic stimulation condition and increased corticosterone. APP/PS1 mice display A β pathology from 4–5 months of age²⁵, in the hippocampus and neocortex, and associated damage to synapses²⁶, and the hippocampus directly regulates the HPA axis^{27–29}. Green and colleagues³⁰ speculated that,

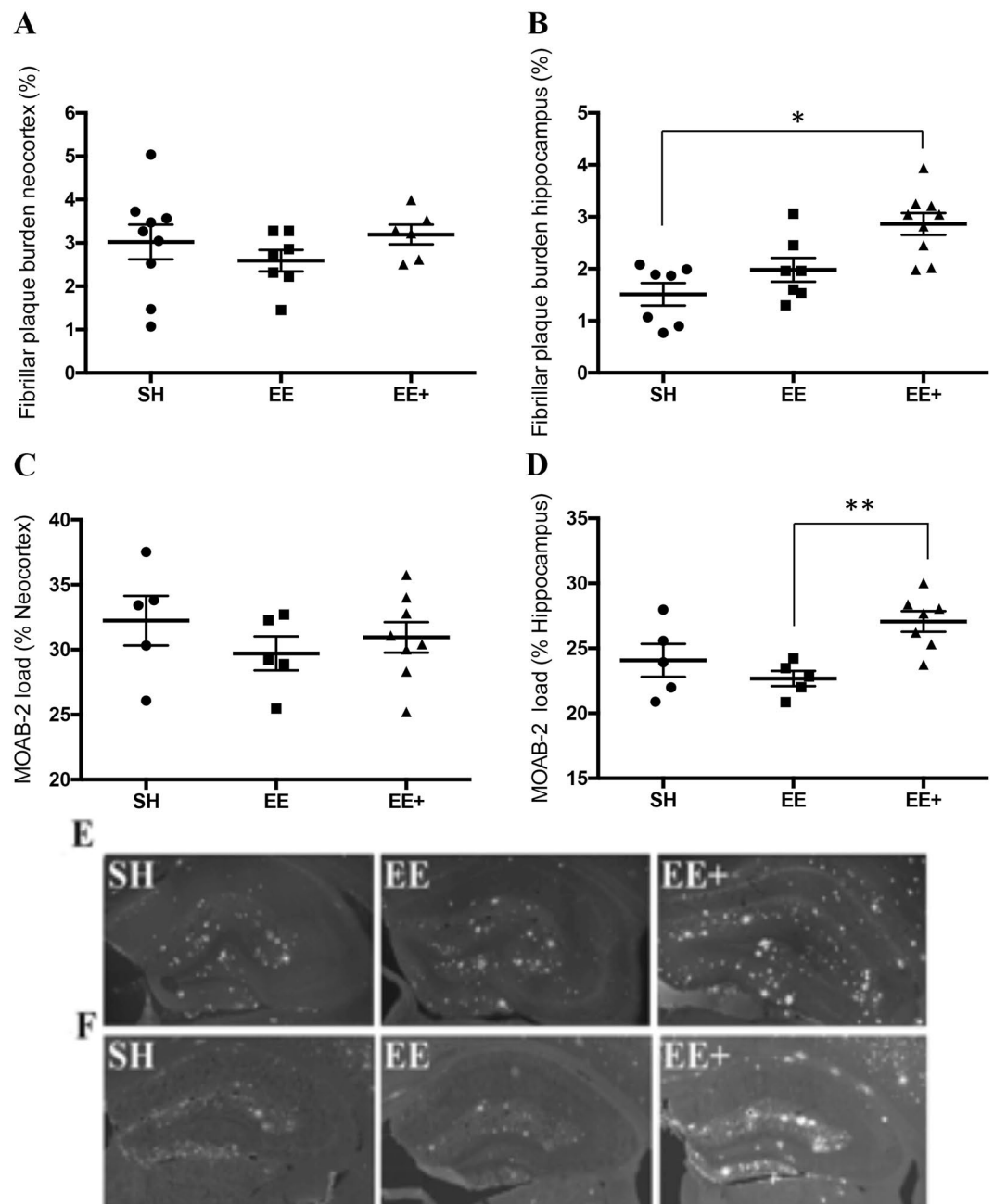


Figure 1. A β plaque load in neocortex and hippocampus in APP/PS1 mice exposed to differential housing. (A) Thioflavin-S staining of fibrillar and dense core A β plaques demonstrated no significant difference in plaque load in neocortex between the housing groups, $F_{(2,19)} = 0.78$, $p = 0.47$. (B) A significant effect of housing was detected on Thioflavin-s plaque load in the hippocampus, $F_{(2,19)} = 4.80$, $p = 0.02$. Bonferroni adjusted *post hoc* tests revealed APP/PS1 mice housed in EE+ had higher hippocampal A β fibrillar plaque burden when compared to those in SH ($p = 0.02$). (C) MOAB-2 immunolabelling demonstrated no significant differences across housing groups in A β_{1-42} load, $F_{(2,18)} = 0.65$, $p = 0.54$. (D) A significant effect of housing condition was detected on hippocampal MOAB-2 load, $F_{(2,18)} = 7.78$, $p = 0.005$. Bonferroni adjusted *post hoc* tests showed EE+ mice to have a significantly higher hippocampal MOAB-2 load relative to mice in SH. SH ($n = 9$), EE ($n = 7$), EE+ ($n = 6$) * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (E) Representative 10x Thioflavin-s staining in hippocampus of APP/PS1 mice for each housing group. (F) Representative 10x MOAB-2 staining in hippocampus of APP/PS1 mice for each housing group.

in AD, glucocorticoid feedback to the hippocampus may be lost, leading to an increased stress response. The increasing levels of glucocorticoid may further accelerate pathological processes, acting as a cycle of disease process progression. Notably, Wt mice did not show increased corticosterone levels after exposure to EE+, and, thus, were not stressed by the periodic stimulation of the EE+ condition. In contrast, FAD gene-linked deposition

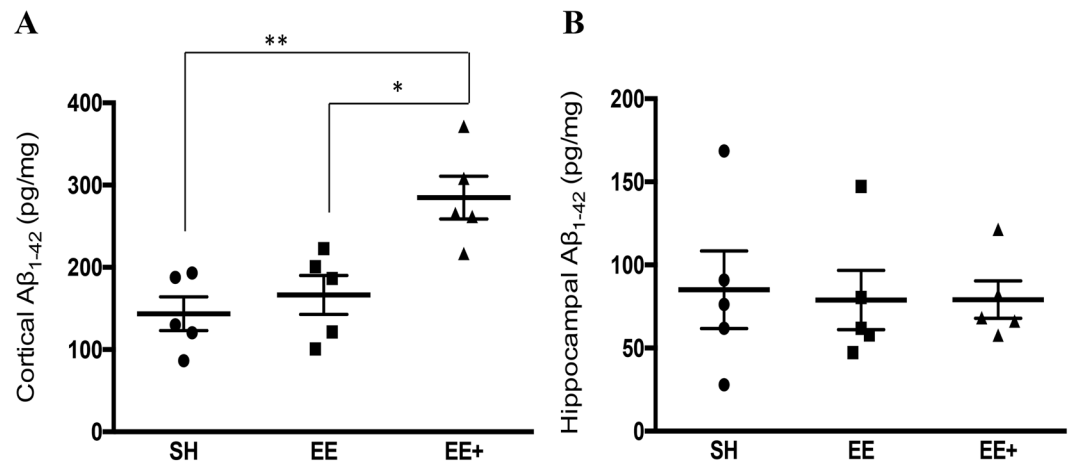


Figure 2. Aβ₁₋₄₂ levels in neocortex and hippocampus in APP/PS1 mice across differential housing conditions. **(A)** Overall, there was a significant effect of housing condition on cortical Aβ₁₋₄₂ levels, $F_{(2,12)} = 10.41$, $p = 0.002$. Follow-up *post-hoc* tests were performed with Bonferroni correction applied. The EE+ group had significantly higher cortical Aβ₁₋₄₂ compared with both SH ($p = 0.003$) and EE ($p = 0.012$). **(B)** No significant differences were detected in hippocampal Aβ₁₋₄₂ levels between housing conditions, $F_{(2,12)} = 0.04$, $p = 0.96$. SH ($n = 5$), EE ($n = 5$), EE+ ($n = 5$) * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

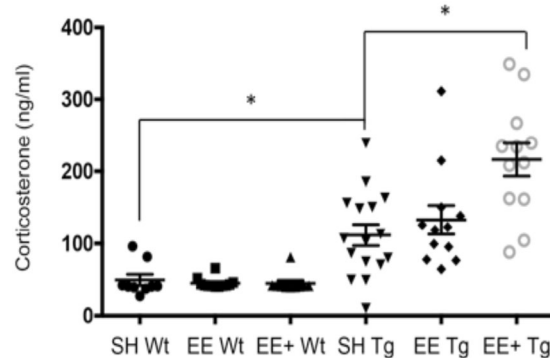


Figure 3. Differential corticosterone levels according to genotype and housing condition. APP/PS1 mice in SH had significantly higher levels of corticosterone in blood serum compared to healthy Wt control mice at 12 months, $t_{(24)} = 2.34$, $p = 0.03$. There was a significant interaction effect of genotype \times housing on levels of corticosterone, $F_{(2,66)} = 3.76$, $p = 0.03$. In Wt mice, there was no significant effect of housing on corticosterone, $F_{(2,28)} = 0.31$, $p = 0.74$. Housing condition produced a significant effect on corticosterone levels in APP/PS1 mice, $F_{(2,38)} = 4.8$, $p = 0.01$. Bonferroni adjusted *post hoc* tests revealed EE+ mice had significantly higher corticosterone levels compared to SH ($p = 0.02$). Moreover, EE+ mice had higher corticosterone levels than mice housed in EE ($p = 0.049$), however, this was no longer significant when Bonferroni correction was applied ($p = 0.057$). SH Wt ($n = 9$), EE Wt ($n = 12$), EE+ Wt ($n = 10$); SH Tg ($n = 17$), EE Tg ($n = 12$), EE+ Tg ($n = 12$) * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

of Aβ in the hippocampus and synaptic damage in transgenic animals may lead to initial dysregulation of the HPA-axis, which contributed to a heightened stress response following periodic stimulation in the EE+ condition. Whereby, this periodic stimulation in EE+ may stimulate a harmful cascade, with subsequent increases in Aβ leading into an increased stress response to EE+, further increasing circulating corticosterone, and further Aβ deposition within the hippocampus.

The finding that elderly who enter a nursing home demonstrate more rapid cognitive decline compared to those who remain in the community³¹, together with the findings of this study, raise questions into the effect of novel environments for people with AD on disease progression. Moreover, the findings presented here bring into question cognitive interventions for the elderly, and for people living with AD in particular. An intervention aimed at delaying the progression of the clinical syndrome, dementia, is urgently needed given the rapidly increasing ageing population. As there are no current efficacious pharmacological treatments available, non-pharmacological interventions such as cognitive stimulation, training, and rehabilitation are of current interest. Such interventions have demonstrated various levels of efficacy, although modest sized effects are typically reported³². To our knowledge, the effects of cognitive intervention on stress in people living with AD has not been examined. Moving forward, this will be an important point of investigation, as cognitive interventions for

people living with AD may have limited beneficial effect, and may potentially induce stress leading to an increased progression of disease processes. Likewise, given that A β pathology may be present in the brain many years before overt dementia symptomology³³, it will be important to examine the role that stress may have in facilitating further pathology, and that primary and secondary prevention strategies that involve cognitive stimulation are designed to minimise elevated stress.

Methods

Animals and EE protocol. In the current study, we used a transgenic mouse model of AD that most closely resembles early-stage AD²⁶. Male mice expressing chimeric mouse/human amyloid precursor protein (APP) and mutant human presenilin 1 (PS1) on C57BL/6 background [B6.Cg-Tg (APP^{swe}, PSEN1^{ΔE9}) 85Dbo/J] (APP/PS1; Jankowsky *et al.*²⁵ and littermate wildtype (Wt) control mice were housed in standard housing (SH) conditions (4–5 mice per 30 × 30 × 14 cm cage, *ad libitum* access to food and water, an igloo, one small wooden stick and one tissue) until 6 months of age. At 6 months, mice were randomly assigned to SH, EE, or EE+ conditions for the following 6 months. Mice assigned to the EE and EE+ housing conditions lived in a larger 60 × 30 × 14 cm cage with the contents of the SH cage and the addition of enrichment objects (wooden and plastic blocks of differing shapes and sizes, platforms, a ball, running wheel, and a mouse hut). The EE+ condition involved the mice being exposed to a larger (36 × 49 × 22 cm) cage three times per week that contained novel enrichment objects (changed weekly). Housing conditions were maintained until the 12-month end-point. All experimental procedures presented here were performed in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, and approved by the University of Tasmania Animal Ethics Committee (A13253).

Tissue collection. Mice were terminally anaesthetized first with gas anaesthesia (isoflurane) followed by sodium pentobarbitone (100 mg/kg delivered intraperitoneally). For histological analysis, animals were perfused transcardially with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffered saline (PBS pH 7.4). Post-mortem brains were transferred to 18% then 30% sucrose solutions overnight. Brains were cut on a cryostat (Leica CM 1850) in 40 μ m coronal sections. APP/PS1 mice used for the quantification of human soluble A β_{42} , were terminally anaesthetized as above, and perfused transcardially with PBS (0.1 M). Post-mortem brains were removed and the cortex and hippocampus were dissected and immediately frozen in liquid nitrogen. Cortex and hippocampal samples were stored at −80 °C for later analysis.

A β fibrillar plaque deposition. Thioflavin-S (Sigma-Aldrich) staining was performed in order to visualise fibrillar and dense-core A β plaques³⁴, ten serial sections evenly spaced throughout the rostrocaudal axis of the brain from bregma 2.0–3.0 mm according to the stereotaxic mouse brain atlas³⁵ were incubated in the solution (0.125% Thioflavine-S diluted in 60% absolute ethanol with 40% 0.01 M PBS) for 3 minutes at room temperature, and washed for 2 × 1 minute in 50% absolute ethanol and 50% 0.01 M PBS solution, followed by 3 × 10 minute washes in 0.01 M PBS. MOAB-2 immunostaining was performed in order to visualize human fibrillar, as well as unaggregated and oligomeric forms of A β_{42} ^{14,36}. Ten serial sections evenly spaced throughout the rostrocaudal axis of the brain from bregma 2.0–3.0 mm were immunolabelled with the MOAB-2 antibody (1:2000; Novus Biologicals), and the labelling was visualized by incubation in Alexa-fluorophore conjugated secondary antibody (1:1000; Molecular Probes, goat anti-mouse IgG2b-546). Sections were mounted using Dako fluorescent mounting medium.

Image acquisition. In order to determine A β plaque load in the neocortex and hippocampus, images were taken on a Leica DM fluorescence microscope on a 10x objective with NIS Elements imaging software. The left side of the cortex was imaged from the midline to the rhinal fissure from bregma 2.0–3.0 mm of 10 sections per animal. Images of the whole hippocampal formation were taken between bregma position −1.22 and −2.46 mm of 3–5 sections per animal. A β plaque load (percentage area Thioflavin-S positive) was calculated by random forest segmentation for one hemisphere of the neocortex and the whole hippocampal formation. Whereby, a custom plugin for ImageJ was applied to the images, which automatically segmented images as plaques or background pixels by random forest classification³⁷. The percent area stained by Thioflavin-S in the neocortex and hippocampus was calculated by dividing the labelled area by the total area analysed.

A β_{42} ELISA. For the quantitation of human A β_{1-42} , a sandwich antibody ELISA was performed according to the manufacturer's instructions (KHB3441, Invitrogen). The right side of the neocortex and hippocampus were homogenized in RIPA buffer (Sigma) containing a protease (Roche diagnostics) and phosphatase inhibitor cocktail (AG Scientific). The samples were centrifuged for 15 minutes at 13000 RPM, rotated for a further 30 minutes, and centrifuged again at 4 °C for 15 minutes at 13000 RPM. The resulting supernatant was removed and stored at −80 °C for protein analysis. The protein concentrations of samples were determined using the Bradford assay. Duplicate neocortex and hippocampus samples from APP/PS1 mice ($n = 5$ /group) were diluted in the buffer provided (1:50). Soluble human A β_{1-42} levels were normalized to total protein levels and expressed as picogram of A β_{1-42} content per milligram of total protein (pg/mg). Optical densities were read at 450 nm on a microplate reader (SpectraMax, Molecular Devices), and concentrations of A β_{1-42} were determined by comparison to the standard curve using a 4-parameter algorithm.

Corticosterone ELISA. In order to determine if there were differences in levels of stress between the housing conditions and genotypes, blood was collected at time of perfusion by cardiac puncture, performed at the start of the dark period (3 p.m.). Duplicate serum samples were diluted at 1:100 in the buffer provided, and levels of serum corticosterone were measured by a commercial ELISA kit (ab108821; Abcam) according to manufacturer instructions. Optical densities were read at 450 nm on a microplate reader (SpectraMax, Molecular Devices),

and concentrations of corticosterone were determined by comparison to the standard curve using a 4-parameter algorithm.

Statistical analysis. Statistical analyses were performed using independent t-test and two-way ANOVA using IBM SPSS (Version 20). A statistically significant two-way ANOVA was followed up by *post hoc* tests. Variables considered were genotype (Wt or Tg) and housing condition (SH, EE, or EE+). Values of $p < 0.05$ for differences between group means were classified as statistically significant. The magnitude of differences between the means were reported as Cohen's d .

Data availability. The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Acknowledgements

The authors would like to thank and acknowledge Graeme McCormack, Justin Dittmann, John McManus, and Aidan O'Mara for technical assistance on this project. This study was funded by the J.O. and J.R. Wicking Trust (Equity Trustees). Mathew J. Summers reports personal fees from Eli Lilly (Australia) Pty Ltd and from Novotech Pty Ltd, outside of the submitted work.

Author Contributions

K.E.S. contributed to the design of the study, wrote initial drafts of the manuscript and undertook the laboratory studies. C.M.F.-M. contributed to the ELISA studies and also to the design of experiments and writing of the manuscript. A.E.K. and J.C.V. contributed to the conceptualization of the project, design of the study and the writing of the manuscript. M.J.S. contributed to the analysis of the study findings and the writing of the manuscript.

Additional Information

Competing Interests: The authors declare that they have no competing interests.

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